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Contribution of basic residues of the A helix of heparin cofactor II to heparin- or dermatan sulfate-mediated thrombin inhibition

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Abstract **Inhibition of thrombin by heparin cofactor II (HCII) is accelerated 1000-fold by heparin or dermatan sulfate. To investigate the contribution of basic residues of the A helix of HCII to this activation, we constructed amino acid substitutions (K101Q, R103L, and R106L) by site-directed mutagenesis. K101Q greatly reduced heparin cofactor activity and required a more than 10-fold higher concentration of dermatan sulfate to accelerate thrombin inhibition compared with wild-type recombinant HCII. Thrombin inhibition by R106L was not significantly stimulated by dermatan sulfate. These results provide evidence that basic residues of the A helix of HCII (Lys^{101} and Arg^{106}) are necessary for heparin- or dermatan sulfate-accelerated thrombin inhibition.**

Key words: Serpin; Heparin cofactor II; Heparin; Dermatan sulfate; Thrombin

1. Introduction

Heparin cofactor II (HCII) is a serine protease inhibitor (serpin) found in human plasma and selectively inhibits thrombin [1]. The rate of thrombin inhibition by HCII is significantly increased in the presence of glycosaminoglycans (GAGs) such as heparin and dermatan sulfate [2]. The heparin or dermatan sulfate binding site of HCII has been localized to the D helix region [3–5]. In another heparin binding serpin, antithrombin (AT), the heparin binding sites are included in the cluster of basic amino acids in the A helix and the D helix [6–9]. Although the role of the basic amino acids in the D helix of HCII in thrombin inhibition stimulated by GAGs has been extensively investigated [10,11], the contribution of basic amino acids in the A helix to GAG-mediated thrombin inhibition has not been examined. Among basic amino acids of the A helix of AT, Arg^{47} is responsible for heparin binding and heparin-mediated thrombin inhibition [6,7]. Since HCII contains Arg^{103} in the position equivalent to Arg^{47} of AT, only the experiment with mutation of Arg^{103} of HCII has been performed. Mutation data have indicated that Arg^{103} of HCII is not critical for heparin binding and inhibition of thrombin by heparin or dermatan sulfate [5].

In this study, we performed competition assays using synthetic peptides corresponding to amino acid residues in the A helix and site-directed mutagenesis of three basic residues (Lys^{101}, Arg^{103}, and Arg^{106}). Based on these experiments, we have demonstrated the roles of basic amino acids of the A helix of HCII in heparin- or dermatan sulfate-mediated thrombin inhibition.

2. Materials and methods

2.1. Materials

Human α-thrombin was purchased from Sigma (St. Louis, MO, USA). Heparin (bovine lung) and dermatan sulfate (pigskin) were from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Chromogenic substrate S-2238 was from Chromogenix (Mölndal, Sweden). Synthetic peptides were obtained from Takara Shuzo Co. Ltd. (Kyoto, Japan). Synthetic oligonucleotides used for mutagenesis were obtained from Genset Oligos Japan (Kyoto, Japan). Heparin-Sepharose CL-6B resin was from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

2.2. Mutagenesis of HCII cDNA

The *Eco*RI fragment containing HCII full-length cDNA (gift from Dr. D.M. Tollefsen, Washington University) was subcloned into the *Eco*RI site of glutathione *S*-transferase (GST)-fusion expression vector pGEX-6P-1 (Amersham Pharmacia Biotech AB). The obtained plasmid then underwent deletion of the 5′-untranslated region and signal sequence region of HCII insert to express wild-type recombinant HCII (rHCII). That is, glycine at the position 1 of HCII follows the cleavage site of the PreScission™ Protease. The construct was designated pGEX-6P-HCWT. The nucleotides at the junction of pGEX-6P-1 and the 5′ end of HCII insert are as follows: CAG*G-GGCCCCTGGGATCCCCGGGGAGCAAAGGC (the codon for Gly^{1} of HCII is underlined and the asterisk represents the cleavage site (Gln*Gly) of the PreScission™ Protease). Mutagenesis yielding Lys^{101} → Gln, Lys^{101} → Glu, Arg^{103} → Leu, and Arg^{106} → Leu was performed using the Quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA); mutagenic primers used in this procedure were, for Lys^{101} → Gln, 5′-GCTTTTTCATGGC*CA*GAGCCGGATC-CAGCG-3′ and 5′-CGCTGGATCCGGCTCT*G*GCCATGAAAAA-GC-3′; for Lys^{101} → Glu, 5′-GCTTTTTCATGGC*GA*GAGCCGGA-TCCAGCGTC-3′ and 5′-GACGCTGGATCCGGCTCT*C*GCCATG-AAAAAGC-3′; for Arg^{103} → Leu, 5′-CATGGCAAGAGCC*T*GATC-CAGCGTCTTAAC-3′ and 5′-GTTAAGACGCTGGATC*A*GGCTC-TTGCCATG-3′; and for Arg^{106} → Leu, 5′-GGCAAGAGCCGGAT-CCAGC*T*TCTTAACATCCTCAACGCC-3′ and 5′-GGCGTTGAG-GATGTTAAGA*A*GCTGGATCCGGCTCTTGCC-3′. The nucleotides in italics indicate the sites leading to codon substitutions. The final constructs were confirmed by sequencing using the chain termination method of Sanger et al. [12].

2.3. Purification of rHCII variants

rHCII was expressed in *Escherichia coli* BL21 cells and purified from the cell lysates according to the purification instructions recom-

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Abbreviations: HCII, heparin cofactor II; rHCII, recombinant heparin cofactor II; serpin, serine protease inhibitor; AT, antithrombin; GAG, glycosaminoglycan

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Table 1
Competition between HCII and synthetic peptides in heparin-mediated thrombin inhibition

Helix	Name of synthetic peptide	Sequence	IC_{50}^{a} (μM)
A	KSR20 (101–120)[b]	KSRIQRLNILNAKFAFNLYR	3.7
D	EIT20 (175–194)	EITTIHNLFRKLTHRLFRRN	18
E	LLD13 (214–226)	LLDFRTKVREYYF	>100
F	KTN15 (242–256)	KTNNHIMKLTKGLIK	>100
H	RVV15 (357–371)	RVVERWQKSMTNRTR	>100

[a]The peptide concentration that blocked 50% of inhibitory activity was measured using inhibitory activity in the absence of peptide as the starting point (0%) and thrombin activity in the absence of HCII as the endpoint (100%). Plots of thrombin activity versus peptide concentration were typically sigmoidal.
[b]The numbers in parentheses represent the positions of amino acid residues corresponding to those of HCII.

mended by Amersham Pharmacia Biotech. Briefly, the cell lysate (from a 2 l culture) suspended in 20 ml of phosphate-buffered saline (PBS)-containing Complete™ Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany) was sonicated and then centrifuged. The supernatant was applied to a GSTrap FF column (5 ml, Amersham Pharmacia Biotech) at 4°C. The column was washed with PBS and then substituted with 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol, pH 7.4 (PreScission™ Protease buffer). The cleavage of rHCII from GST by PreScission™ Protease (Amersham Pharmacia Biotech) was performed on the column. A 4.5 ml portion of PreScission™ Protease buffer containing 100 U of PreScission™ Protease was injected onto the column and the system was incubated at 4°C overnight. Prior to elution, a 1 ml GSTrap FF column (pre-equilibrated with PreScission™ Protease buffer) was connected downstream to the above 5 ml GSTrap FF proteolytic cleavage column. rHCII cleaved from GST was eluted immediately upon flow startup with PreScission™ Protease buffer. The purity of the final preparations was assessed by SDS–PAGE on 7.5% polyacrylamide gels stained with Coomassie blue. Protein concentrations were determined with a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA, USA).

2.4. Inhibition of thrombin by rHCII variants

In the absence of GAG, the second-order rate constants (k_2) for thrombin inhibition by various rHCII variants were determined under pseudo first-order conditions as described previously [13]. The effect of heparin or dermatan sulfate on inhibition of thrombin by rHCII variants was determined by incubating 250–500 nM rHCII variants with 1.9 nM thrombin and various concentrations of heparin or dermatan sulfate in 200 μl of assay buffer (50 mM Tris–HCl, 150 mM NaCl, and 0.1% bovine serum albumin, pH 8.4). After 5 min incubation at room temperature, 50 μl of 1 mg/ml S-2238 was added, and the residual thrombin activity was determined by measuring the change of absorbance at 405 nm. Peptide competition assays were performed by the addition of synthetic peptides (0.1–100 μM) to the assay mixture containing 2.5 μg/ml heparin or 25 μg/ml dermatan sulfate.

2.5. Heparin-Sepharose affinity chromatography

A 1 ml heparin-Sepharose column was equilibrated with 50 mM NaCl, 50 mM Tris–HCl, pH 7.4. Each purified rHCII preparation was diluted with 50 mM Tris–HCl, pH 7.4, to the NaCl concentration of 50 mM, and applied to the column. The column was then washed with 5 ml of the starting buffer and eluted with a 20 ml gradient of 50–500 mM NaCl in 50 mM Tris–HCl, pH 7.4. Fractions of 1 ml were collected and their absorbances were measured at 280 nm. The denatured rHCII was detected in the column flow-through. The salt concentration corresponding to peak elution was the average of three runs.

Table 2
Heparin affinity of rHCII variants

rHCII variant	Heparin affinity[a] (mM NaCl)
Wild-type	220
K101Q	150
R103L	200
R106L	210

[a]Heparin affinity is given as the salt concentration required for elution from a 1 ml column of heparin-Sepharose.

3. Results

3.1. Competition assays with synthetic peptides

Table 1 lists the sequence of synthetic peptides corresponding to amino acid residues in the helix structure of HCII (helices A, D, E, F, and H). The peptides were tested for their ability to compete with wild-type rHCII for heparin binding in thrombin inhibition assays. Table 1 presents the results of these experiments, which are expressed as the concentrations of peptide (IC_{50}) required to block half of the thrombin inhibition activity of wild-type rHCII in the presence of heparin. Among these peptides, KSR20 and EIT20 competed with wild-type rHCII in the thrombin inhibition assays, with KSR20 being more effective in doing so. In contrast, RVV15 did not interfere with heparin-catalyzed thrombin inhibition, although this peptide had four Arg residues and one Lys residue. The results suggest that the specific sequence of the peptide, not merely its charge, is important for heparin binding. In the presence of dermatan sulfate, KSR20 also blocked inhibition of thrombin, with an IC_{50} of 17 μM. These results suggest the possibility that heparin and dermatan sulfate may bind to positively charged amino acid residues included in residues 101–120 of HCII. We then prepared three rHCII mutants, K101Q, R103L, and R106L, which replaced Lys^{101} with Gln, Arg^{103} with Leu, and Arg^{106} with Leu, respectively.

3.2. Binding of rHCII variants to heparin-Sepharose

Heparin-Sepharose chromatography was performed to determine the relative affinities of the rHCII variants for heparin (Table 2). Wild-type rHCII was eluted from the heparin-Sepharose column with a peak at 220 mM NaCl. R103L and R106L did not affect the ionic strength at which they eluted from heparin-Sepharose. The results indicate that the positive charge on the amino acid at position 103 or 106 of HCII is not required for heparin binding when assessed by affinity chromatography. In contrast, K101Q was eluted from the

Table 3
Second-order rate constants for inhibition of thrombin by rHCII variants in the absence of a GAG

rHCII variant	k_2 (M^{-1} min^{-1})	%
Wild-type	8.09×10^3	100
K101Q	8.40×10^3	104
R103L	12.4×10^3	153
R106L	10.6×10^3	131

Assays were performed as described in Section 2. k_2 values presented are the mean of at least three determinations.

heparin-Sepharose column with a peak at 150 mM NaCl, suggesting that K101Q has a decreased affinity for heparin.

3.3. Inhibition of thrombin by rHCII variants in the absence or presence of heparin or dermatan sulfate

As shown in Table 3, second-order rate constants (k_2) of these rHCII mutants for inhibition of thrombin in the absence of GAG were similar to the value obtained for wild-type rHCII. These findings provide evidence that the rHCII proteins are folded properly, and that these reactive sites, in particular, are intact. The k_2 value obtained for wild-type rHCII (8.09×10^3 M^{-1} min^{-1}) was 10-fold lower than the value reported previously [13]. The differences in rate constants may be related to differences in expression vector system and purification methods. The lower values may result from the imprecision in the determination of rHCII concentration due to the denatured protein.

The effects of heparin or dermatan sulfate on the extent of inhibition of thrombin by rHCII variants are shown in Fig. 1. In these experiments, wild-type rHCII inhibited 50% of the thrombin activity in the presence of 0.35 μg/ml heparin or 4.3 μg/ml dermatan sulfate. Most importantly, K101Q greatly reduced heparin cofactor activity, which is consistent with the observation that K101Q has a lower affinity than wild-type rHCII for heparin-Sepharose. In contrast, R103L and R106L required only three-fold higher concentrations of heparin than wild-type rHCII to inhibit 50% of thrombin activity. These observations suggest that Lys^{101} is essential for heparin-dependent thrombin inhibition.

In the presence of dermatan sulfate, thrombin inhibition by R106L was not stimulated (Fig. 1, bottom). K101Q required at least a 10-fold higher concentration of dermatan sulfate to accelerate inhibition of thrombin in comparison with wild-type rHCII. These results suggest that Arg^{106} and Lys^{101} are necessary for dermatan sulfate-dependent thrombin inhibition. R103L, however, had no effect on the ability of either heparin or dermatan sulfate to accelerate the reaction, consistent with the findings of Blinder et al. [5]. These results indicate that Lys^{101} is required for heparin- and dermatan sulfate-dependent thrombin inhibition, and that Arg^{106} is necessary for dermatan sulfate- but not heparin-mediated thrombin inhibition. This suggests that the binding sites of heparin or dermatan sulfate in the A helix of HCII are not identical.

To confirm the importance of the positive charge at Lys^{101}, we constructed the mutant, K101E, which featured substitution of positively charged amino acid by negatively charged amino acid. In the absence of GAG, the k_2 value of K101E for thrombin inhibition was 5.14×10^3 M^{-1} min^{-1}, which was slightly lower than that of wild-type rHCII. As shown in Fig. 1, no acceleration of thrombin inhibition by K101E was observed with either heparin or dermatan sulfate. These findings indicate that the positive charge at Lys^{101} is essential for acceleration of thrombin inhibition of HCII by heparin or dermatan sulfate.

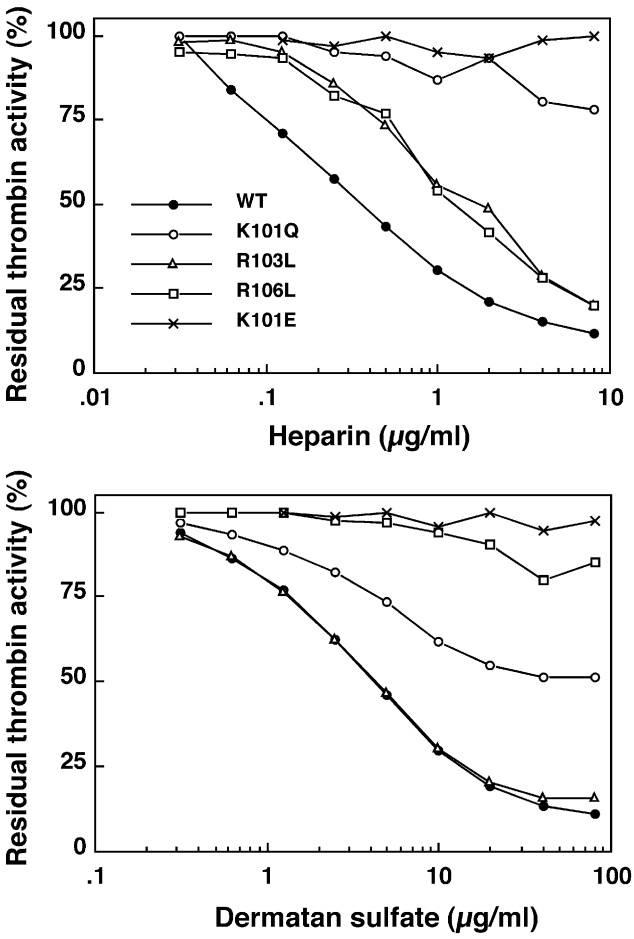

Fig. 1. Effects of heparin and dermatan sulfate concentrations on inhibition of thrombin by rHCII variants. Thrombin (1.9 nM) was incubated with 250–500 nM rHCII variants in the presence of various concentrations of heparin and dermatan sulfate. After 5 min, residual thrombin activity was measured by adding S-2238 and expressed as a percentage of the control (in the absence of GAG). Top is heparin; bottom is dermatan sulfate.

4. Discussion

HCII is a serpin and is further classified as a heparin-binding serpin [14]. The current model of the mechanism of activation of HCII suggests that the amino-terminal acidic domain of HCII is freed from intramolecular interactions upon heparin binding and interacts with exosite I on thrombin [15–17]. The binding site of heparin to HCII is included in the D helix and has been extensively investigated. In another serpin, AT, the heparin binding site has been localized to the A helix and the D helix and is known to undergo a conformational change upon binding of heparin. Moreover, it has been reported that the heparin binding site of protein C inhibitor is included in the H helix but not the D helix [18]. In HCII, the cluster of basic amino acids lies within the A and H helices in addition to the D helix [19]. In this study, a synthetic peptide (KSR20) corresponding to the A helix of HCII (residues 101–120) interfered with heparin- or dermatan sulfate-catalyzed thrombin inhibition. The ability of KSR20 to interfere with the reaction was stronger than that of a synthetic peptide (EIT20) corresponding to the D helix of HCII (residues 175–194). The results obtained from peptide competition assays suggested that heparin or dermatan sulfate might bind to amino acid residues included in the A helix of HCII.

K101Q exhibited a significant decrease in heparin-Sepharose affinity. K101Q almost lost the ability to accelerate thrombin inhibition in the presence of heparin, and required more than 10-fold higher concentrations of dermatan sulfate to accelerate thrombin inhibition in comparison with wild-

type rHCII. Furthermore, K101E exhibited no acceleration of thrombin inhibition by either heparin or dermatan sulfate. These observations suggest that the binding sites of HCII for heparin and dermatan sulfate contain Lys^{101}, and that the positive charge at Lys^{101} is essential for GAG-accelerated thrombin inhibition.

R106L could not exhibit acceleration of thrombin inhibition with dermatan sulfate but did not affect the heparin-accelerated reaction. Furthermore, R106L was eluted from heparin-Sepharose at the same ionic strength as wild-type rHCII. These findings suggest that Arg^{106} plays a major role in the stimulation of dermatan sulfate-mediated thrombin inhibition but does not interact with heparin. The binding sites in the A helix of HCII for the two GAGs are thus apparently not identical, as found for the D helix of HCII [10].

R103L inhibited thrombin in the same manner as wild-type rHCII in the presence of either heparin or dermatan sulfate. In AT, Arg^{47} is essential for acceleration of thrombin inhibition by heparin. However, mutations in the equivalent position in HCII (Arg^{103}) produced different results. In the amino acid sequence alignment of HCII and AT, the amino acid corresponding to Lys^{101} in HCII is not found in AT [20]. Although there are no three-dimensional structural data for HCII, it is possible that the side chain of Lys^{101} is exposed to the surface of the HCII molecule and the side chain of Arg^{103} is oriented toward the inside of the molecule.

Liaw et al. have reported that release of the amino-terminal domain of HCII by charge neutralization at five basic residues of the D helix is insufficient to fully stimulate inhibition of thrombin [11]. It is possible that Lys^{101} and Arg^{106} are related to the additional intramolecular interactions that constrain the amino terminus. In addition, as elucidated for AT, HCII may also undergo a conformational change of the reactive center upon binding of GAG to the basic residues of the A and D helices of HCII.

In summary, the basic residues of the A helix in HCII, Lys^{101} and Arg^{106}, contribute to acceleration of thrombin inhibition by GAGs. Our results have further defined the specific basic residues within HCII required for heparin- or dermatan sulfate-accelerated inhibition of thrombin.

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